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14. ABSTRACT The goal of this research is to firmly establish the mechanism of androgen receptor (AR)-JunD heterodimer induction of the SSAT gene leading to oxidative stress that contributes to the development and progression of prostate cancer (PCa), and to identify small molecules that specifically inhibit this AR-JunD interaction and prevent development/progression of PCa in pre-clinical models. Data from this research will identify the most efficacious drug to be further developed in preclinical toxicity testing and clinical trials for PCa that fall beyond the scope of this proposal. Significant findings during Year 2 of the research include: identification of two non-antiandrogenic AR-JunD inhibitors that significantly inhibit ROS production and androgen-dependent and -independent growth in PCa cells; and determination that the lead compound is orally bioavailable and therefore a promising clinical drug candidate to be further tested for efficacy against preclinical animal models of PCa as proposed.

15. SUBJECT TERMS androgen receptor, JunD, SSAT, oxidative stress, ChIP, Gaussia luciferase reconstitution, high throughput screen, small molecule inhibitors, human prostate carcinoma cells, pharmacokinetcs, prostate cancer xenograft and transgenic mouse models efficacy

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INTRODUCTION

We hypothesize that activated androgen receptor (AR) forms a heterodimer with JunD and this AR-JunD heterodimer induces SSAT gene expression leading to polyamine oxidation and consequent production of excess reactive oxygen species (ROS) in prostate cells. Excess ROS, in turn, contribute to the development and progression of prostate cancer (PCa). The purpose of this research is to firmly establish the mechanism of the induction of SSAT by the AR-JunD complex that leads to an excess ROS production in PCa cells, identify small molecules that specifically inhibit the AR-JunD interaction downstream of androgen activation of AR in this pathway, validate the activity of the inhibitors against ROS production and growth in PCa cells, and determine the chemotherapeutic/chemopreventive efficacy of the lead inhibitors against pre-clinical mouse models of PCa. Data from this research will identify the most efficacious drug to be further developed for preclinical toxicity testing and clinical trials for PCa that fall beyond the scope of this proposal. This report summarizes the progress that has been achieved toward completing the proposed aims.

BODY

The following are the data collected with respect to tasks listed in our statement of work (SOW):

Task 1. Establish the mechanistic pathway for androgen-induced SSAT expression in CaP cells (months 1-18):

1.1. Create mutant SSAT promoter-luciferase reporter constructs (months 1-4).
1.2 Transiently transfect mutant constructs into cells and compare 1) SSAT promoter activity by luciferase reporter assay and 2) reduction in androgen-induced ROS production by DCFH dye oxidation assay (months 4-12).
1.3 Perform ChIP assay to identify the AR-JunD binding sequence in most and least effective mutants (months 12-18).

This task was largely completed in the first year. To recap, cell culture studies on LNCaP cells transiently transfected with SSAT promoter-luciferase reporter constructs, including three differently truncated promoter constructs and the full length promoter construct, showed enhancement of SSAT promoter activity following androgen stimulation for all but the truncated promoter with a 659 bp lead sequence. The data suggested a 3-dimensional structure of the promoter that may hide certain sections in the SSAT promoter that are important for androgen induction of SSAT. Our previously published chromatin immunoprecipitation (ChIP) data showed that a 77 bp section between 550-650 bp lead sequence is the JunD binding domain of the SSAT promoter in the presence of androgen [1].

To complete this task in Year 2, we introduced point mutations in the AP1/JunD binding sequences within the SSAT promoter sequence and repeated the studies with these point-mutated constructs. No difference in SSAT promoter activity following androgen stimulation was observed, thus we concluded that the point mutations did not disturb JunD binding. Since the point mutations had no effect, we did not pursue the ChIP assay in cells transfected with these constructs as proposed.

Task 2. Screen a small molecule library to identify inhibitors of AR-JunD interaction (months 1-15):

2.1 Optimize the GL-reconstitution screening assay conditions using the "hit" obtained from the partial screening assay performed in preliminary studies (months 1-4).
2.2. Perform the optimized screen on the 14,400 compound Maybridge HitFinder library (months 4-12).

2.3. Eliminate false positive "hits" by performing the positive control (SMAD3-PKB) interaction screen (months 6-15).

This task was completed in the first year. To recap, after standardizing a more efficient high throughput screen (HTS) assay using the NCI Diversity Set library that had been used in the preliminary studies presented in the grant proposal, we completed our screen for new "hits" in a 25,000 compound Life Chemicals library, which yielded 13 small molecule drug-like compounds that specifically inhibited the AR-JunD interaction by at least 30%, and were thus advanced to Task 3.

These compounds are currently being submitted for patent process. Details of these compounds will be made available under confidentiality upon request.

Task 3. Select inhibitors that act downstream to AR activation and validate compounds for activity against CaP cells (months 8-28):
3.1 Test compounds for their ability to bind AR using an AR-LBD binding fluorescence polarization assay (months 8-26).
3.2 Test compounds for their ability to inhibit the translocation of AR-JunD to the cell nucleus (months 8-26).
3.3 Test compounds for their ability to inhibit growth of CaP cells and block androgen-induced ROS production in CaP cells (months 10-28).

Following our determination in Year 1 that 12 of the 13 compounds are non-antiandrogenic, we proceeded with cell culture studies in Year 2 to determine the activity of the small molecule inhibitors against androgen-induced ROS production and androgen-dependent and -independent prostate cancer cell growth. We chose the compounds to be tested in the full series of ROS and growth cell culture studies based on percent inhibition of the AR-JunD interaction by the compounds in the HTS assay: 7 of the 12 non-antiandrogenic compounds were selected for these studies based on ≥ 50% inhibition of AR-JunD in the HTS.

DCF and DNA assays were used to measure the amount of ROS production and growth, respectively, in both androgen-dependent LNCaP and androgen-independent LNCaP C4-2 cell lines following our established methods [2-4]. Briefly, for ROS studies, LNCaP cells were grown in F1C4 +/- androgen analog methyltrienolone (R1881) for androgen induction of ROS as previously published [2-4]. For growth studies cells were grown in DMEM containing either 5% FBS for androgen-dependent (AD) growth or under androgen deprivation in 1% FBS and 4% charcoal-stripped serum (F1C4) for androgen-independent (AI) growth. Dose response studies of the 7 selected compounds were performed under these conditions to determine 50% effective (EC50) or inhibitory (IC50) concentrations for each drug against androgen-induced ROS production, AD growth and AI growth. Results for the 7 compounds are summarized in **Table 1**. Two compounds, GWARJD10-001 and GWARJD14-001, showed significant activity against androgen-induced ROS production and AD growth in LNCaP cells as well as LNCaP C4-2 AI growth (**Figure 1**). Both compounds had EC50s of 25 μM or less and IC50s of 10 μM or less, which are considered clinically relevant concentrations and thus these compounds were prioritized for further studies.

Table 1. Ranking of GWARJD small molecule compounds based on cell culture studies				
Drug Name	ROS Block ~EC50	AD Growth Inhibition ~IC50	AI Growth Inhibition ~ IC50	Ranking
GWARJD10-001	10uM	4uM	4uM	1
GWARJD11-001	> 25uM	> 25uM	> 25uM	
GWARJD12-001	> 25uM	> 25uM	> 25uM	
GWARJD13-001	> 25uM	10uM	25uM	
GWARJD14-001	10uM	10uM	25uM	2
GWARJD18-001	> 25uM	25uM	> 25uM	
GWARJD19-001	> 25uM	> 25uM	> 25uM	

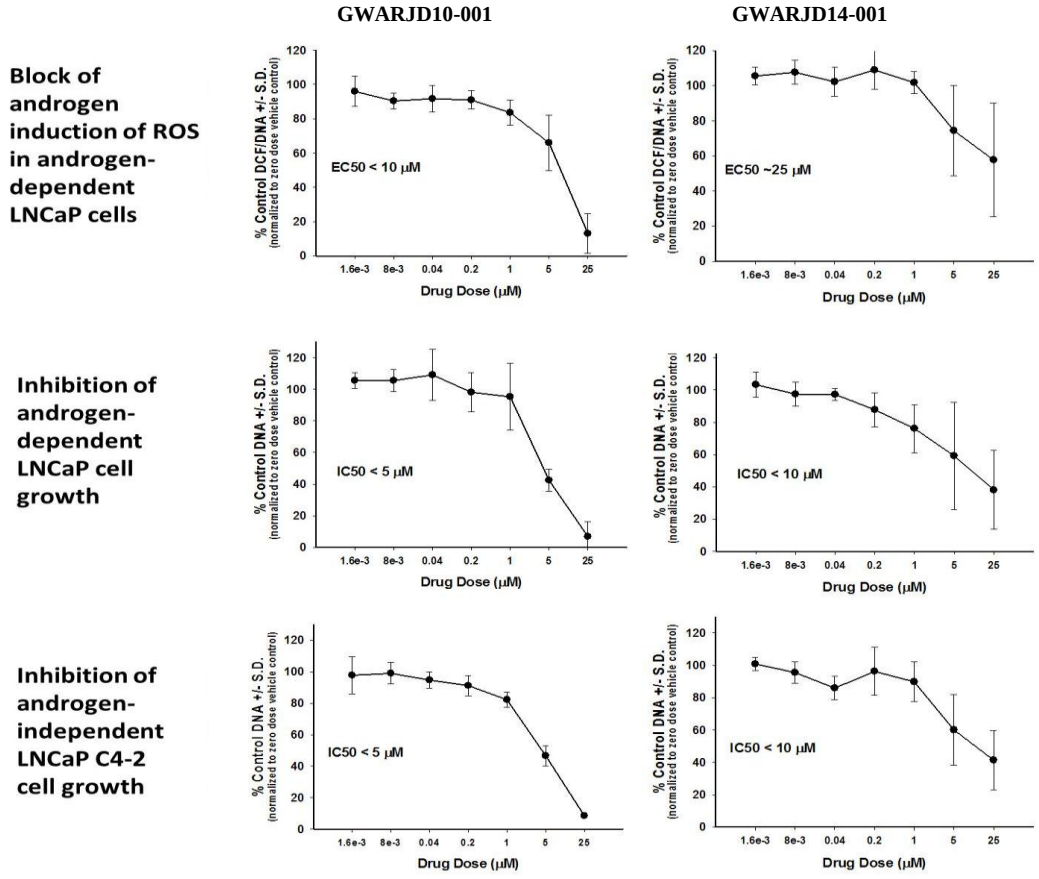

Figure 1. GWARJD10-001 and GWARJD14-001 block androgen induction of ROS and inhibit both androgen-dependent and androgen-independent prostate cancer cell growth. Cells were grown in DMEM containing either 5% FBS for androgen-dependent (AD) growth or under androgen deprivation in 1% FBS and 4% charcoal-stripped serum (F1C4) for androgen-independent (AI) growth. For ROS studies, cells were grown in F1C4 +/- 1 nM androgen analog methyltrienolone (R1881) for androgen induction of ROS as previously published. Following 4 days to treatment, DCF and DNA assays were used to measure the amount of ROS production and growth, respectively, in both androgen-dependent LNCaP and androgen-independent LNCaP C4-2 cell lines following our established methods [2-4]. N=6 per data point, experiments were performed in duplicate.

Immunocytochemistry (ICC) studies to determine the ability of the top compounds to inhibit the nuclear translocation of AR-JunD complex are ongoing. We established a quantitative ICC analysis using new, sophisticated technology - a Nuance™ fluorescence microscope (Caliper/PerkinElmer) equipped with spectral deconvolution and AUC integration software - available in the UWCCC TRIP lab. Initial studies on compounds GWARJD10-001 and GWARJD14-001 were performed in LNCaP cells treated with ROS-inducing 1nM R1881 synthetic androgen and varying doses of compound. GWARJD10-001 showed significant activity against androgen-stimulated co-translocation of AR and JunD to LNCaP cell nuclei, while GWARJD14-001 was not effective at the same concentrations (**Figure 2**). However, AR staining was less than optimal in this study, and we are currently working with the UWCCC TRIP lab to improve the AR staining in this analysis in order to finalize these studies.

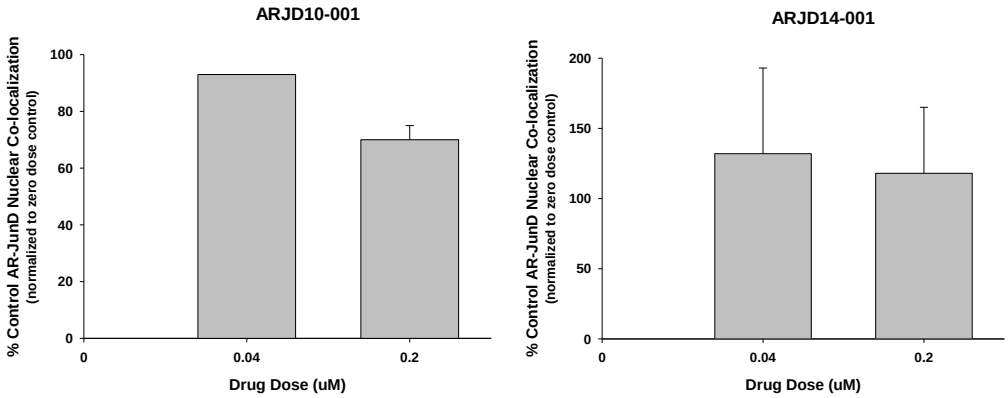

Figure 2. Quantative immunocytochemistry shows androgen induced co-translocation of JunD and AR into LNCaP cell nuclei is blocked by ARJD10-001. LNCaP cells were cultured in F1C4 medium with 1nM R1881 +/- drug for 4 days, then assayed via immunocytochemistry (ICC) using anti-JunD and anti-AR antibodies. The nuclear and cytoplasmic fluorescence was quantitated using the Nuance™ fluorescence microscope equipped with spectral deconvolution and AUC integration software. Amount of JunD co-localized with AR *per cell* in cells treated with R1881 with or without pretreatment with the agents is calculated from:
$F_{co\text{-}loc} = F_{JunD}^{AR}/F_{DAPI}$

Where F_{JunD}^{AR} = Fluorescence of JunD ICC in the area defined by AR ICC fluorescence and F_{DAPI} is the fluorescence of DAPI stain

The data at various doses of the selected agents were presented as the per cent of that in cells treated with androgen alone. The experiment was performed in duplicate.

Task 4. Select the lead drug candidate for future clinical testing by comparing efficacy of 2 to 3 drug candidates in mouse models of CaP (months 6-36).

4.1 Screen potential drug candidates for oral bioavailability, pharmacokinetics and maximum tolerated dose in mice (months 6-28).

4.2 Determine efficacy of drug candidates against a CaP cell xenograft model in nude mice (months 12-32).

4.3 Determine efficacy of drug candidates against the TRAMP mouse model of CaP (months 15-34).

4.4 Complete a statistical comparison of the efficacy of drug candidates against both CaP animal models to select the lead drug for further pre-clinical testing and future clinical trial (months 34-36).

Using the methods of testing and LC-MS protocol standardized in Year 1, we initiated animal pharmacokinetics (PK) studies on the lead (top-ranked) compound, GWARJD10-001, in Year 2. We determined solubility of the compound in our standard administration vehicle, 0.9% saline. A preliminary PK / toxicity study established that a single dose of 5 mg/kg in 6%DMSO saline by

either intra venous (IV) or oral administration was well-tolerated and yielded plasma levels at 30 minutes post administration of 3.2nM for IV and .05nM for oral dosing (**Table 2**). Thus the compound was determined to have oral bioavailability, and is therefore an ideal candidate for clinical application. Additional PK and maximum tolerated dose (MTD) studies for a daily oral dosing regimen of GWARJD10-001 are underway, to be followed by efficacy studies in xenograft and transgenic mouse models of cancer as planned. We will perform additional solubility / formulation studies on GWARJD10-001 to potentially improve the solubility (to reduce percent DMSO in the vehicle for higher dose levels) and determine stability in solution for long-term administration studies. Solubility / formulation analysis of GWARJD14-001 is also planned.

Table 2. GWARJD10-001 levels in plasma over time following 5 mg/kg single dose

Mice were harvested at 30 minutes, 2 hours or 1 week following the single dose administration (N=2 per condition). Plasma was extracted and LC-MS carried out by a standardized gradient of 2% acetic acid water and 2% acetonitrile.

	30 minutes	2 hours	1 week	
Oral	.05 nM	.02 nM	0	
IV	3.2 nM	1.9 nM	0	

KEY RESEARCH ACCOMPLISHMENTS

- Established mutant SSAT promoter-luciferase reporter constructs
- Identified relatively more important sequences in SSAT promoter for androgen induction of SSAT
- Optimized the high throughput screen for 384-well plate based assay
- Completed screening on a 25,000 compound Life Chemical (LC) library
- Identified 13 compounds as true “hits”
- Identified 12 of the 13 “hits” as non-antiandrogenic compounds
- Standardized conditions for testing the compounds for efficacy against growth and ROS production in androgen-dependent (AD) and androgen-independent (AI) human prostate carcinoma (PCa) cells in the presence or absence of androgen
- Completed cell culture studies on 7 of the non-antiandrogenic LC compounds (selected for testing based on > 50% in HTS): determined EC50s for block of ROS production, IC50s for AD and AI PCa cell growth
- Identified 2 compounds with significant activity against ROS production and PCa cell growth based on EC50s and IC50s; prioritized them for further study
- Standardized a quantitative ICC method for determining ability of compounds to reduce AR-JunD nuclear co-localization
- Performed ICC studies on top 2 compounds
- Standardized protocol for development of LC-MS analysis and testing of compounds for oral biovailability and pharmacokinetics
- Developed a LC-MS method for the lead (rank 1) compound
- Performed a preliminary PK/toxicity analysis for the lead compound at 5 mg/kg oral versus iv (single dose); determined oral bioavailability and single dose tolerance

REPORTABLE OUTCOMES

- Kegel S, Mehraein-Ghomi F, Reuter Q, Schmidt J, Church D, Hoffmann FM, Basu H, and Wilding G. Targeting a novel pathway for prostate cancer therapy [abstract]. In: Proceedings of the 102nd Annual Meeting of the American Association for Cancer

Research; 2011 Apr 2-6; Orlando, Florida. Philadelphia (PA): AACR; 2011. Abstract nr 2583 (see Appendix 1)

- Schmidt J, Kegel S, Mehraein-Ghomi F, Saphner E, Reuter Q, Church D, Hoffmann FM, Basu H, and Wilding G. Inhibiting Androgen Receptor and JunD protein interaction to prevent prostate cancer progression [abstract]. In: Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31 - Apr 4; Chicago, Illinois. Philadelphia (PA): AACR; 2012. Abstract nr 2844 (see Appendix 2)
- Plasmids developed: Four different mutant SSAT promoter-luciferase reporter constructs with various degrees of truncation of the SSAT promoter connected to a luciferase reporter in a pGL4 vector; Ap1/JunD sequence point-mutated SSAT promoter-luciferase reporter constructs
- SBIR Phase I grant proposal submitted December 2010, resubmitted December 2011: "Androgen Receptor-JunD Complex Inhibitors to Prevent Prostate Cancer Progression" (Colby Pharmaceutical Company PI Hirak Basu, UW-Madison PI George Wilding)

CONCLUSION

We have established that point mutations in the AP1/JunD consensus sequence (TGA/TCA) do not affect the binding of JunD to the SSAT promoter under androgen stimulation. However, removing bigger sequences in the SSAT promoter did significantly affect the androgen-stimulated SSAT promoter activity, and indicated a 3-dimensional structure where certain sequences important for androgen-induced SSAT activation may be hidden.

We have successfully identified drug-like small molecules that specifically inhibit the AR-JunD interaction downstream of androgen activation of the AR, and validated significant activity against androgen-induced ROS production and androgen-dependent and –independent growth in human PCa cells by two of these AR-JunD inhibitors. One of these compounds has also shown significant activity against AR-JunD co-translocation to the nucleus in the PCa cells. We have also determined that this compound is orally bioavailable. Thus, we have identified at least one small molecule AR-JunD inhibitor that shows significant promise as a potential new agent for human PCa therapy. Planned studies in preclinical animal models should establish a lead compound to translate to preclinical and clinical development to treat early-stage recurrent prostate cancer patients, who have no approved therapy and represent a long unmet medical need.

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transgenic adenocarcinoma of the mouse prostate model. Cancer Res. 2009; 69:7689-95. PMCID: PMC2756327.

APPENDICES

Appendix 1: AACR 2011 abstract
Appendix 2: AACR 2012 abstract

APPENDIX 1

Targeting a novel pathway for prostate cancer therapy

Stacy Kegel, Farideh Mehraein-Ghomi, Quentin Reuter, Joseph Schmidt, Dawn Church, F. Michael Hoffmann, Hirak Basu, George Wilding. University of Wisconsin- Madison, Madison, WI
In: Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research; 2011 Apr 2-6; Orlando, Florida. Philadelphia (PA): AACR; 2011. Abstract nr 2583

Development of an effective therapy to prevent prostate cancer (PCa) progression to castrate-resistant PCa (CRPCa) remains an unmet medical need, mainly due to a poor understanding of the mechanism of PCa progression. Reactive oxygen species (ROS) are produced in high amounts in PCa cells and play a major role in PCa development and progression. We have published that activated androgen receptor (AR)-JunD complex induces spermidine/spermine N1-acetyl transferase (SSAT), the first and regulatory enzyme in a major polyamine catabolism pathway that yields over-production of ROS specifically in the polyamine-rich PCa cells. Our recent data further suggest an intriguing mechanism of PCa progression, where AR-JunD induced SSAT expression and consequent upregulation of the transcription factor NF-□B may set up an autocrine feed forward loop of SSAT-ROS-NFκB-SSAT that can sustain ROS production and PCa cell proliferation in the absence of androgen. A focus of our current research is to identify compounds that specifically target and block steps in this novel pathway downstream to AR activation and thereby have potential to be new targeted therapeutic agents to prevent PCa progression to CRPCa in early stage progressing PCa patients with minimal side effects. In the research presented here we utilized a novel high throughput screen (HTS) assay to find compounds that prevent the initiating AR-JunD interaction step in this ROS generating pathway and tested their efficacy in PCa cells. A high throughput assay based on Gaussia Luciferase enzyme reconstitution via protein-protein interaction was used to screen the NCI diversity set library of drug like molecules to identify inhibitors of the AR-JunD interaction. Selected hits were further screened to determine their ability to bind to the AR using a published fluorescence polarization assay in order to categorize the molecules as non-antiandrogens or antiandrogens. As we intend to target the pathway downstream of AR activation, we focused on non-antiandrogens, further testing them for efficacy against androgen induced ROS generation and effect on cell growth in PCa cells in vitro by our published DCFH dye oxidation and DNA fluorescence assays. Of the 14 hits from the HTS assay of the NCI diversity set library, nine small molecules were chosen based on their drug-like chemical characteristics for further studies along with two synthesized analogs of one of the selected small molecules. We have categorized six agents as non-antiandrogens and five as having some antiandrogenic activity. Our ROS assay identified a lead non-antiandrogen compound that blocks androgen induced ROS production in PCA cells at less than 1uM concentration. This agent also significantly inhibited androgen-independent growth of PCa cells at 4uM. Data for all compounds will be presented.

APPENDIX 2

Inhibiting Androgen Receptor and JunD protein interaction to prevent prostate cancer progression

Joseph Schmidt, Stacy Kegel, Farideh Mehraein-Ghomi, Elizabeth Saphner, Quentin Reuter, Dawn Church, F. Michael Hoffmann, Hirak S. Basu, George Wilding. University of Wisconsin Carbone Cancer Center, Madison, WI

In: Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31 – Apr 4; Chicago, Illinois. Philadelphia (PA): AACR; 2012. Abstract nr 2844

Development of an effective therapy to prevent prostate cancer (PCa) progression to castrate-resistant PCa (CRPCa) remains an unmet medical need, mainly due to a poor understanding of the mechanism of PCa progression. Reactive oxygen species (ROS) are produced in high amounts in PCa cells and play a major role in PCa development and progression. Our published data show that activated androgen receptor (AR)-JunD complex induces the enzyme spermidine/spermine N1-acetyl transferase(SSAT), the first and a regulatory enzyme in a major polyamine catabolism pathway that causes over-production of ROS specifically in the polyamine-rich PCa cells. A focus of our current research is to identify compounds that specifically target and block steps in this novel pathway downstream to AR activation. Such compounds have the potential to become new targeted therapeutic agents with minimal side effects for preventing progression to CRPCa in patients with early stage progressing Pca. Here we present data on drug-like small molecule inhibitors of the AR-JunD interaction that initiates this ROS-generating pathway in PCa. A novel high throughput assay based on Gaussia Luciferase enzyme reconstitution via protein-protein interaction was used to screen NCI Diversity and Life Chemicals libraries of drug-like small molecules to identify inhibitors of the AR-JunD interaction. The 22 selected hits were categorized as antiandrogens or non-antiandrogens through the use of a published fluorescence polarization AR binding competition assay. Eighteen were identified as non-antiandrogens, acting downstream of AR activation by androgen, of which 14 were chosen for further study. In vitro studies entailed the measurement of the ability of the compounds to inhibit androgen-independent and dependent growth as well as AR-induced ROS in human PCa cells. The top three compounds exhibited significant inhibition of growth and androgen-induced ROS at concentrations of 5uM or less. Immunocytochemistry (ICC) studies were performed to determine the ability of the selected compounds to specifically inhibit the AR-JunD co-translocation to the nuclei. Based on the cell culture and ICC data, lead compounds were selected for pharmacokinetics studies to determine oral bioavailability. Detailed pharmacokinetic data of selected top compounds showing bioavailability after intravenous and oral administration will be presented.